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PRINCIPAL INVESTIGATOR: Zhihong Lu, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-2010

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Zhihong Lu, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Alabama at Birmingham
Birmingham, Alabama 35294-2010

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Dihydropyrimidine Dehydrogenase (DPD) is the initial, rate-limiting enzyme in catabolism of 5-fluorouracil (5-FU), one of the most widely used chemotherapeutic agents in the treatment of breast cancer.The purpose of this project is to improve 5-FU-based chemotherapy by monitoring DPD. DPD activity in peripheral blood mononuclear cells (PBM-DPD) was determined in 360 patients with breast cancer, with the mean PBM-DPD (0.26±0.01 nmol/min/mg protein) being significantly lower than that observed in female controls (0.44±0.02 nmol/min/mg protein, $p<0.005$). ANOVA analysis examining the significance of differences in DPD activity among various groups indicated that only disease difference (breast cancer vs. normal subjects) was significant after adjustments for race and age. Significant lower DPD activity in patients with breast cancer may predispose to 5-FU-associated toxicity. Preliminary analysis suggested that breast cancer patients with DPD activity lower than 0.10 nmol/min/mg protein may have greater risk in developing 5-FU-associated toxicity. In the present study, 21 (5.8%) patients were considered to be DPD deficiency, indicating that this pharmacogenetic syndrome may be more common than anticipated. These results should be useful in the future to individualize the 5-FU dosage and thus reduce the risk of 5-FU-associated toxicity and/or improve therapeutic efficacy in patients with breast cancer.

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Zhihong Lu 09/09/97

PI - Signature Date

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INTRODUCTION

I. BACKGROUND

5-Fluorouracil (5-FU) is one of the most widely used anticancer drugs (1-2), ranking in the top three anticancer drugs prescribed in the U.S. (3). It is frequently used in the treatment of breast cancer (4). In general, 5-FU-associated toxicity occurs in the gastrointestinal mucosa and bone marrow (4,5) and, less frequently, in the neurologic system presenting as cerebellar ataxia and somnolence. Like many other antineoplastic drugs, 5-FU has a relatively narrow therapeutic index, such that toxicity is likely to increase as the dose is escalated. The biochemical mechanism of 5-FU-associated toxicities is thought to be related to its anabolic pathway, in particular, inhibition of thymidylate synthase and incorporation into RNA and DNA (1-2). However, 5-FU catabolic pathway may have a major role in 5-FU toxicity since more than 85% of administered 5-FU is metabolized by the catabolic pathway. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting step in 5-FU catabolism (6), which is important in regulation of 5-FU metabolism, determining the availability of 5-FU for anabolism and further determining 5-FU therapeutic effects and/or toxicities.

Several clinical studies indicate the importance of DPD as a major determinant of 5-FU toxicity. Co-administration of thymidine with 5-FU (leading to release of thymine that competes DPD with 5-FU) has been shown to induce unexpected life-threatening toxicity (7-9). Pharmacokinetic studies of patients receiving 5-FU by continuous infusion demonstrated that plasma 5-FU levels varied throughout the day with a circadian pattern (10). Subsequent study examining patients receiving 5-FU by continuous infusion demonstrated that plasma 5-FU levels had a circadian variation that varied inversely with circadian variation in DPD from peripheral blood mononuclear (PBM) cells, suggesting that the plasma 5-FU levels were regulated by DPD (11). The relationship between DPD levels and 5-FU pharmacokinetics was also demonstrated by Fleming et al (12) in patients receiving 5-FU continuous infusion.

The importance of DPD in 5-FU pharmacokinetics and toxicity is further demonstrated in patients with DPD deficiency, a recently defined pharmacogenetic syndrome (13-18). Following 5-FU-based chemotherapy, these patients developed profound toxicity and eventually died. More importantly, in our earlier studies (14-17), ten of the 11 patients were women with either breast or colorectal cancer. In view of this striking sex difference in patient studies from our laboratory (14-17) and others (13), as well as the recently suggested sex-related difference in 5-FU clearance (18), it is important to determine if there is sex difference in DPD activity and in 5-FU toxicity by a large scale prospective study. Recently, we determined the characteristics of DPD activity and established a baseline for this enzyme in the general population (17).

There are an increasing number of genetic polymorphisms recognized for drug metabolizing enzymes that may produce not only altered drug metabolism but also increased drug toxicity. Pharmacogenetic syndromes have now been described for many different types of drugs including cancer chemotherapy drugs (19). While decreased drug metabolism may result in altered pharmacokinetics and pharmacodynamics and hence increased toxicity with various classes of drugs, it can be particularly striking with cancer chemotherapy drugs since the therapeutic index is typically narrow. In the present project, we have been studying the pharmacogenetic syndrome of DPD deficiency, potentially accounting for many of the cases of severe 5-FU toxicity, including death, seen in the clinic. The presence of genetic polymorphism in the population due to the presence of unknown molecular alteration(s) in DPD suggests that if DPD can be assessed prior to

therapy severe toxicity may be avoided in the future. Although the mechanisms are unclear, the frequency of DPD deficiency in women and breast cancer patients appears to be higher than that in general population.

II. PURPOSE AND SIGNIFICANCE OF THE PROJECT

The long term objective of this project is to improve 5-FU chemotherapy in cancer patients through a better understanding of the genetic polymorphism of DPD and its role in determining 5-FU toxicity. Knowledge acquired from this project should be useful in the future to predict which patients may be susceptible to severe 5-FU toxicity, permitting modification of drug dose before chemotherapy. Specific objectives include:

1.) Determine in a prospective study the relationship between DPD activity and 5-FU toxicity.

Hypothesis 1- *-DPD has a major role in determining 5-FU toxicity with decreased enzyme activity being associated with greater toxicity. DPD activity in breast cancer patients may be used in predicting 5-FU toxicity and permitting modification of drug dose of 5-FU in chemotherapy of breast cancer.*

2.) Determine biochemical properties of DPD from peripheral blood mononuclear cells (PBM-DPD) of normal and deficient individuals.

Hypothesis 2-- *The mechanisms responsible for DPD deficiency may be related to DNA, RNA and protein levels. Comparison of DPD from deficient patients with DPD from normal individuals should provide insight into the mechanism of genetic polymorphism of DPD.*

As noted earlier, 5-FU continues to be one of the most widely used anticancer drugs (1-2), ranking in the top three anticancer drugs prescribed in the U.S. (3). It is one of the major chemotherapeutic agents in the treatment of breast cancer(4). However, the mechanisms responsible for 5-FU toxicity are not completely understood. Although toxicity from 5-FU is generally manageable, severe and life threatening toxicity does occur. As shown above DPD is the initial rate limiting enzyme in 5-FU catabolism and hence can ultimately regulate the amount of 5-FU available for anabolism. Furthermore, we have shown that there is evidence for variation in enzyme in the population, and that certain individuals who experience severe toxicity after 5-FU treatment are deficient in DPD activity. Preliminary studies indicate that DPD activity in breast cancer patients is lower than the general population. In addition, most DPD deficient patients identified by us and others were women. Furthermore, the frequency of DPD deficiency in breast cancer patients appears to be much higher than in the general population. Thus it appears highly desirable to determine in a prospective study the relationship between DPD activity and toxicity in patients receiving 5-FU, and to determine the mechanisms responsible for DPD deficiency.

It is believed that liver is the major site for 5-FU catabolism, however, the risk of invasive procedure to measure liver DPD activity is not justified. Previous studies suggested that PBM-DPD can be used as a marker for liver-DPD activity (17,18). Population characteristics of PBM-DPD have been described in several recent studies (10,17,18) in normal volunteers and cancer patients; however, there is limited information available in patients with breast cancer in the USA. The present study was undertaken to characterize DPD activity in patients with breast cancer as well as the frequency of DPD deficiency in this population.

METHODS AND MATERIALS

Chemicals and Radiochemical

5-FU, BSA, NADPH, and Histopaque were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]-5-FU (25 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The purity of unlabeled and labeled 5-FU was confirmed by HPLC (20) to be greater than 99%. All other solvents and reagents were purchased in the highest grade available.

The major buffer (buffer A) used in both the enzyme preparation and DPD assay contained 35 mM potassium phosphate, pH 7.4, 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol. Since it is light-sensitive and unstable with long-term storage, NADPH, the critical cofactor in the enzyme reaction was freshly prepared.

Determination of PBM-DPD Activity in Breast Cancer Patients

Cancer Patients. In collaboration with the Oncology Clinic in the University of Alabama at Birmingham, breast cancer patients who were seen in the clinic and treated or to be treated with 5-FU (in combination with other agents) were assayed for DPD activity. Consent was obtained from each patient. Clinical data were also collected for further analysis.

Blood Collection and Isolation of PBM Cells. Blood samples (25 ml) were drawn from a peripheral vein into heparinized tubes and then loaded onto a centrifuge tube containing 15 ml Histopaque. After centrifugation at 500 x *g* for 30 min at 25°C, the PBM cell fraction was carefully removed and washed 3 times with PBS. Contaminating red blood cells were hypotonically lysed. The resulting PBM cells were used in the subsequent enzyme assay.

Preparation of PBM cytosol. Fresh PBM cells were suspended in buffer A then placed in an ice bath and lysed by sonication (3 times 10 sec with 30 sec interval between sonication). After centrifugation at 14, 000 x *g* for 15 min at 4°C, the supernatant was removed and used in the subsequent enzyme assay. Using the method of Bradford (21), the amount of protein in the sample was determined prior to enzyme assay in order to add appropriate amount protein into the reaction mixture.

Enzyme Assay DPD activity was determined by radioassay, measuring the catabolites of 5-FU formed by reversed-phase HPLC (17, 20). The reaction mixture contained 200 μM NADPH, 20 μM [^{3}H]-5-FU, buffer A, and enzyme solution (250-1000 μg total protein) in a final volume of 1 ml. The mixture was incubated at 37°C and 175 μl of the reaction sample was taken out at various times (5, 10, 20, 30, 60 min) and mixed with the same volume of ice-cold ethanol to stop the reaction. The mixture was then kept in a freezer (-20°C) for 30 min and subsequently filtered through a 0.2 μm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis.

Reversed-Phase HPLC Analysis Separation of 5-FU and its catabolites was performed by reversed-phase HPLC using a Hewlett-Packard 1050 HPLC system equipped with a spectrometric detector and chromatographic terminal (HP 3396 Series II Integrator). Two Hypersil 5 μm columns (Jones Chromatography, Littleton, CO) were used in tandem as the stationary phase. The columns were eluted at a flow rate of 1.0 ml/min with the mobile phase containing 1.5 mM potassium phosphate, pH 8.0, with 5 mM tetrabutylammonium hydrogen sulfate (17,20).

Fractions (1 ml) were collected into 7-ml scintillation vials, using a Redifrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ) and were mixed with 5.5 ml scintillation solvent. The radioactivity in each fraction was quantitated by liquid scintillation spectrometry (Beckman LS 6000). Under these conditions, typical retention times for dihydrofluorouracil and 5-FU were 9 and 21 min, respectively.

Determination of DPD Activity in Normal Liver and Tumor Tissues

Liver Tissues from Donors. Human livers were obtained from the National Disease Research Interchange (NDRI), Philadelphia, PA, and the University of Alabama Comprehensive Cancer Center Tissue Procurement Shared Facility. The protocol used in this study was approved by both the university Institutional Review Board and NDRI. In the present study, 138 human liver samples (83 males and 55 females) from donors with normal liver function were collected. The 138 human liver samples were free of liver disease. These liver samples were collected in a 2-year period. Liver samples were quickly frozen and kept in -70°C freezer until use. No significant systemic difference in DPD activity was found for samples obtained at various times over a 2-year period.

Patients and Samples. This part of study was carried out in collaboration with the Department of Medical Oncology, Cancer Hospital, Sun Yat-sen University of Medical Sciences in Guangzhou, China. Fifty cancer patients were enrolled in the present study. All patients were diagnosed with primary hepatocellular carcinoma that was relatively small and surgically removable. All patients gave informed consent to participate in this study. The protocol used in this study was approved by the University Institutional Review Board of Sun Yat-sen University of Medical Sciences. The hepatocellular carcinoma and the adjacent uninvolved liver tissue samples (about 10 g) were removed during surgery. Each sample was divided into two parts, one being used for pathologic evaluation and the other for DPD analysis. Specimens for pathologic evaluation were fixed in formalin solution. The samples for DPD analysis were frozen immediately and stored in liquid nitrogen until transported in dry ice to our laboratory in USA. These samples were then stored at -70 °C until DPD analysis. Permission to import biologic samples was obtained from US Center for Disease Control (CDC).

Sample Preparation. The slowly-thawed liver tissues were washed with ice-cold physiologic saline (0.9% NaCl), weighed, minced, and homogenized in 4 volumes of buffer A. The resulting homogenate was centrifuged at 100,000 x *g* for 60 min at 4 °C. The cytosolic fraction (supernatant) was removed and used in the subsequent analyses. DPD activity was determined immediately following the supernatant preparation. Prior to enzyme assay, the amount of protein in each sample was determined by the method of Bradford (21) in order to add the appropriate amount of protein to the enzyme reaction. The experimental conditions for enzyme reaction and HPLC analysis were essentially the same as described above.The reaction mixture in buffer A contained 20 μM [^{3}H]-5-fluorouracil, 200 μM NADPH, and sample solution (1 mg of total protein) in a final volume of 2 ml. The mixture was incubated at 37 °C and 350 μl aliquot was sampled at various times points (5, 10, 15, and 20 min) and mixed with the same volume of ice-cold ethanol to stop the reaction. The mixture was then kept in a freezer (-20 °C) for at least 30 min and subsequently centrifuged and filtered through a 0.2 μm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis.

Calculation of DPD Activity

For each sample, 5 determinations were run at various incubation times. After HPLC

analysis, the amount of 5-FU catabolites at each time point was quantitated. The data was plotted using products formed (y) versus time (x) to calculate the slope of the reaction (products formed/min) by linear regression analysis. The slope was then divided by the amount of protein added to obtain the final result (DPD activity expressed as nmol/min/mg protein). For samples from cancer patients with DPD deficiency or liver samples with very low DPD activity, at least two separate assays were performed.

As demonstrated in our previous studies (9, 17), the radioassay was sensitive and accurate in determination of DPD activity in peripheral blood mononuclear cells and in liver samples. Using the assay conditions described above, the variations of inter- and intra-assay were found to be less than 5%. For samples with extremely low enzyme activity, at least two separate assays were performed to verify the results.

Immunoblot Analysis (Western Blot)

The primary antibody used in the study was the purified rabbit polyclonal antibody generated against human liver dihydropyrimidine dehydrogenase (9). A 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the freshly prepared 100,000 *g* liver supernatant, using a 1.0 mm thick, 7% (w/v) polyacrylamide gel containing 0.375 M Tris-HCl (pH 8.8) and 0.1% sodium dodecyl sulfate. Samples were mixed with an equal volume of sample buffer (0.0625 M Tris-HCl, pH 6.8; 10% glycerol; 0.2% sodium dodecyl sulfate (w/v); 80 mM 2-mercaptoethanol) and then boiled for 5 min. Electrophoresis was performed at a constant current of 30 mA for 45 min at 25 °C. The proteins were transferred from the gel to a nitrocellulose filter following the method of Towbin et al (22). Following incubation overnight at 4 °C with the primary antibody (diluted 1:10000) in a 120 mM borate-saline solution containing 1% (w/v) bovine serum albumin, pH 8.5, the nitrocellulose filter was washed with borate-saline containing 0.5% (v/v) Tween 20 and then incubated with a secondary, alkaline phosphatase-labeled goat anti-rabbit antibody. The location of immunoreactive proteins on the nitrocellulose filter was detected in a 0.1 M sodium carbonate buffer (100 ml), pH 9.5, containing 30 mg nitro blue tetrazolium (added as a 1 ml solution dissolved in 70% dimethylformamide) and 15 mg 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (added as a 1 ml solution dissolved in 100% dimethylformamide).

Statistical Analysis

Mean DPD activity (and S.D. or S.E.) was calculated for each group by age, gender, and race. The differences of DPD activity among the groups by gender, age, and race were analyzed by ANOVA. To determine the distribution pattern in the general population, probability testing was used.

RESULTS

I. PBM-DPD Activity in Breast Cancer Patients

PBM-DPD Activity in the Breast Cancer Patients Population.

Using freshly prepared PBM cell samples, DPD activities of 360 patients with breast cancer were determined. The population characteristics of this study are summarized in Table 1. The mean PBM-DPD activity in patients with breast cancer was 0.265 nmol/min/mg protein with highest and lowest values being 0.571 and 0.013 nmol/min/mg protein, respectively. The distribution of PBM-DPD activity in this population is shown in Fig. 1. Statistical analysis by probability testing indicated that human PBM-DPD activity follows a normal distribution.

PBM-DPD Activity in Breast Cancer Patients Grouped by Age and Race.

The mean PBM-DPD activities in each group by age are shown in Table 1 and Fig. 2. Analysis of different age groups indicated that PBM-DPD activities in breast cancer patients age 40 and above were slightly higher than that observed in patients less than 40 (Table 1, Fig. 2). However, these differences were not statistically significant. The mean PBM-DPD activities in each group by race are shown in Table 1. Statistical analysis indicated that there was no significant difference of PBM-DPD activity between Caucasians and African Americans although the mean PBM-DPD activity in African Americans was slightly lower than that in Caucasians.

PBM-DPD Activity in Breast Cancer Patients Compared with Healthy Volunteers.

As illustrated in Fig 1, although the distribution pattern of PBM-DPD Activity in patients with breast cancer was essentially the same as seen in the general female population (18), the distribution and mean activity were shifted to the left, indicating that mean PBM-DPD activity in the breast cancer patient population was significantly lower than that observed in female controls ($P<0.01$, Table 1). Further examination of the affect of disease, race, and age on PBM-DPD activity by ANOVA analysis revealed that only disease difference (cancer vs. normal subjects) was statistically significant after adjustments for race and age. Preliminary analysis of a recently initiated study of colon rectal cancer patients also suggested that mean PBM-DPD activity in breast cancer patients was lower than that observed in patients with colon rectal cancer ($P<0.05$, Fig. 3), although the number colon rectal cancer patients is still relatively small (N = 22).

Frequency of DPD Deficiency in Patients with Breast Cancer.

Twenty-one (5.8%) patients were classified as DPD deficient (their PBM-DPD activities were less than 0.1 nmol/min/mg protein). Initial analysis of the clinical data suggested that the frequency of 5-FU-associated toxicity was greater in patients with PBM-DPD activity lower than 0.10 nmol/min/mg protein than those with higher DPD activity.

II. Population Distribution of Liver DPD Activity in General Population

Population Distribution of Liver DPD Activity.

Using frozen liver samples, dihydropyrimidine dehydrogenase activities of 138 donor livers collected consecutively over the preceding 24 months were determined. The population characteristics of this study are summarized in Table 2. Distribution of human liver dihydropyrimidine dehydrogenase activity is shown in Fig 4. Statistical analysis by probability testing indicated that human liver dihydropyrimidine dehydrogenase activity follows a normal distribution (Fig 5). In order to examine potential differences in gender, age and race, further statistical analyses were carried out. The mean liver dihydropyrimidine dehydrogenase activities in each group by gender, age, race are shown in Table 2. Results from ANOVA analyses are shown in Table 3. Mean dihydropyrimidine dehydrogenase activity in females was significantly higher than that observed in males ($p<0.005$). Although dihydropyrimidine dehydrogenase activity was higher in Caucasians compared to African Americans, the difference was not statistically significant. Analysis of different age groups indicated that dihydropyrimidine dehydrogenase activities in individuals age 30-40 yrs and 40-50 yrs were slightly higher than other groups (Table 2), particularly in females (Fig 6). How ever, these differences were not statistically significant. Further examination of affect of gender, race, and age on dihydropyrimidine dehydrogenase activity by ANOVA indicated that only gender difference was statistically significant after adjustments for race and age (Table 3).

Correlation between Enzyme Activity and the Amount of DPD Protein in the Liver.

Using affinity-purified polyclonal antibody against human dihydropyrimidine dehydrogenase, Western blot analysis demonstrated that dihydropyrimidine dehydrogenase protein was significantly decreased in liver cytosol from the two samples with extremely low enzyme activity compared with samples with normal activity. Representative Western blot analyses were shown in Fig 7A, B, C. As illustrated in Fig. 7B, where an equal amount of protein was loaded onto the gel, the dihydropyrimidine dehydrogenase protein band for two liver samples had a very low density. The correlation analysis between dihydropyrimidine dehydrogenase activity and the density of dihydropyrimidine dehydrogenase protein band on western blot analysis is shown in Fig 8. These results demonstrate a relationship between dihydropyrimidine dehydrogenase activity and the amount of dihydropyrimidine dehydrogenase protein in the liver.

III. DPD Activity in Surgical Hepatoma and Liver Tissues in Chinese Cancer Patients

Pathological Confirmation of Normal Liver and Hepatoma Samples.

Pathological evaluation for each normal liver and hepatoma sample was performed under microscope in the Department of Pathology at the Sun Yat-sen University of Medical Sciences. Forty-seven out of fifty samples were confirmed to be normal liver tissue. The other three samples were shown to be hepatic cirrhosis. However, the DPD activity in three samples with cirrhosis was not statistically different from that in normal liver samples (data not shown). All the tumor samples were shown to be primary hepatoma, nodular type, and grade I to grade III based on WHO standard classification.

Population Distribution of DPD Activities in Normal Liver and Hepatoma Tissues.

Dihydropyrimidine dehydrogenase activity of 50 pairs of normal liver and tumor tissues

were quantified in a Chinese population cancer patients with hepatoma. The population characteristics of this study are summarized in Table 4. Distribution of DPD activities in both normal liver and tumor tissues is shown in Fig 9. Statistical analysis by probability testing indicated that DPD activities in both normal human liver and hepatoma tissues follow a normal distribution, with DPD activity in hepatoma being significantly lower than that in normal liver (paired T test, $P< 0.01$). Of note, a small proportion of normal liver and hepatoma samples had very high DPD activity (Fig. 9). Further statistical analyses showed that the mean normal liver and hepatoma DPD activities between groups by gender and age had no significant difference. However, in each subgroup, the mean hepatoma DPD activities were consistently significantly lower ($P<0.01$) than their mean normal liver DPD activities (Table 4, Fig. 10).

Correlation between Normal Liver DPD Activity and Hepatoma DPD Activity.

The correlation analysis between normal liver DPD activity and hepatoma DPD activity is illustrated in Fig. 11. No significant linear correlation was observed in the present study ($r=0.196$, $P>0.05$).

DISCUSSION

Task 1. Determine in a prospective study the relationship between DPD activity and 5-FU toxicity

The major purpose of the present project is to characterize the distribution pattern of PBM-DPD activity in breast cancer patients, to establish the relationship between DPD activity and 5-FU-associated toxicity, and to improve 5-FU-based chemotherapy in breast cancer patients by monitoring DPD activity.

Results from the present study demonstrated that PBM-DPD activity in breast cancer patients generally follows a normal distribution as seen in normal population (17). However, the major finding of the present study is that mean PBM-DPD activity in breast cancer patients was significantly lower than that observed with the general population. This difference (cancer vs. normal subjects) was statistically significant after adjustments for race and age. In an NIH/NCI funded study, we will continue the clinical study examining PBM-DPD activity in breast cancer patients, including collection of clinical data, e.g., disease status, response, and toxicity. Due to the nature of study design of the project (blinded study), we will not be able to determine the relationship between DPD activity and 5-FU-associated toxicity until the end of the clinical study.

Thus far, we have determined DPD activity of 360 cancer patients, demonstrating that the methods used in monitoring DPD activity is sensitive, accurate, reproducible and can be used in the future large-scale screening in cancer patients. Our study has shown that DPD deficiency is not rare (about 3-5% in breast cancer patients). Monitoring DPD activity prior to use of 5-FU will have significant impact on reducing 5-FU-associated toxicity and improving therapeutic effects by adjusting the dose to be administered.

A number of drug metabolizing enzymes have genetic polymorphisms (19). To evaluate the distribution pattern of DPD activity and determine if a genetic polymorphism for DPD exists, several studies have now been undertaken in normal subjects and cancer patient populations

(11,12,17,18,23,24). Most of the previous studies utilized a small population without balance in the number of subjects in each subgroup by gender, age, and race. A recent study in our laboratory with 124 subjects (45% males and 55% females) demonstrated a normal distribution of PBM-DPD activity, with an approximate 4-fold difference in enzyme activity (17). No significant differences in the enzyme activity were observed related to gender, age, or race in the general population (17). In a study of cancer patients, Milano *et al.* (18) reported a possible influence of gender on 5-FU clearance and suggested that it may be related to variations in DPD activity. However, more recent reports from the same laboratory showed no significant gender difference in DPD activity (12,25). In the present study, we reported preliminary results of a cohort study of DPD activity in breast cancer patients. PBM-DPD activity was determined in a larger population (360 female breast cancer patients). The distribution pattern of PBM activity was similar to the general population (17). However, the mean enzyme activity was significantly lower than that observed in the general population (17) and lower than that observed in patients with colorectal cancer. Prospective studies of the relationship between DPD activity and 5-fluorouracil-associated toxicity and/or response are continuing.

Task 2 Determine biochemical properties of DPD from peripheral blood mononuclear cells (PBM-DPD) of normal and deficient individuals.

In the initial design of the proposal, we would like to concentrate on the PBM-DPD to elucidate the mechanisms responsible for DPD deficiency. In earlier studies of the project and other projects in this laboratory, we have shown: (1) the correlation between DPD activity and DPD protein in normal human liver; (2) initial evidence for the correlation between DPD activity and DPD protein in peripheral blood mononuclear cells; and (3) initial evidence for the correlation between liver DPD and PBM DPD activity in patients. However, due to the limited amount of PBM-DPD protein available for biochemical and molecular studies, and the importance of liver DPD in 5-FU metabolism, as well as the abundant liver DPD protein available, we decided to use liver sample to carry out most of biochemical studies of DPD.

The population distribution of liver DPD activity has not been known until recently. In a previous study (26), a 288-fold variation in human liver DPD activity was observed, presumably due to variation in the quality of liver tissue preparation. Results from the first year study of the project demonstrated that liver DPD activity generally follows a normal distribution. Slight differences among race, gender, and ages were observed (27). Gender difference in liver DPD activity was shown to be statistically significant (27). Of note, 4 liver samples had very low enzyme activity (<0.05 nmol/min/mg protein) and 3 liver samples had very high enzyme activity (>0.85 nmol/min/mg protein). Although the importance of extremely low DPD activity has been shown in cancer patients with deficiency of this enzyme, the significance of very high enzyme activity has not been clear but may have a role in the poor response to 5-FU treatment due to increased catabolism of the drug.

We also carried out a study to determine DPD activity in normal and tumor tissues, in collaboration with the Department of Medical Oncology, Cancer Hospital, Sun Yat-sen University of Medical Sciences in Guangzhou, China. One of the advantages of the study is that we could determine the normal and tumor tissue simultaneously to establish the relationship between normal and tumor tissues. Fifty cancer patients were enrolled in the present study. All patients were diagnosed with primary hepatocellular carcinoma that was relatively small and surgically removable. Each sample was divided into two parts, one being used for pathologic evaluation and the other for DPD analysis. Statistical analysis by probability testing indicated that DPD activities in both uninvolved human liver and carcinoma tissues follow a normal distribution, with DPD activity

in tumor being significantly lower than that in uninvolved liver (paired t-test, $P < 0.01$). Further statistical analyses showed that the mean DPD activities in uninvolved liver and hepatocellular carcinoma among groups classified according to gender and age were not significantly different. However, in each subgroup, the mean tumor DPD activities were consistently significantly lower than that of the mean uninvolved liver DPD activities. Of note, mean liver DPD activity (0.45±0.02) was higher that in normal population (0.37 ± 0.02). The mechanism for the difference is not clear.

In a continuing effort to study DPD in this laboratory, we have generated a specific polyclonal antibody against human DPD. Using this antibody, both decreased liver DPD activity and decreased protein corresponding to this enzyme were observed in cancer patients identified to be deficient of this enzyme (17). Studies have now shown the usefulness of the polyclonal antibody in quantitating DPD activity in various tissues including liver (27), peripheral blood mononuclear cells (28), and fibroblasts (29). Using a larger sample size in the present study, we further established the relationship between DPD activity and the amount of DPD protein, providing the insight of DPD deficiency and the basis for the future clinical use of DPD antibody to quantify DPD activity in cancer patients.

CONCLUSIONS

Studies in the project have demonstrated that:

1) DPD activity in peripheral blood mononuclear cells in the breast cancer population generally follows a normal distribution with slight differences in age and race;

2) Significantly decreased DPD activity was observed in breast cancer patients compared to normal population and colon rectal cancer patients;

3) Liver DPD activity in the general population and the cancer population generally follows a normal distribution with slight differences in gender, age and race;

4) Significantly decreased DPD activity was associated with decreased enzyme protein; and

5) DPD activity in tumor tissue is significantly lower than normal liver tissues.

Further studies are needed to determine the mechanisms responsible for lower DPD activity in breast cancer patients, the frequency of DPD deficiency in the other cancer patient population, the relationship between DPD activity and 5-FU effectiveness and/or toxicity, the relationship between DPD activities in peripheral blood mononuclear cells and the liver, and the molecular basis for DPD deficiency.

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Table 1. Dihydropyrimidine Dehydrogenase Activity in Peripheral Blood Mononuclear Cells (PBM-DPD) in Breast Cancer Patients

		DPD Activity (nmol/min/mg protein)			
		Patients with Breast Cancer		Controls[a]	
Group		n	Mean ± S.E.	n	Mean ± S.E.
Total		360	0.265 ± 0.006*	68	0.443 ± 0.016
African American		48	0.243 ± 0.014*	28	0.460 ± 0.026
Caucasian		312	0.270 ± 0.006*	40	0.431 ± 0.020
Age (yr)	20-			22	0.402 ± 0.025
	30-	35	0.230 ± 0.015*	22	0.481 ± 0.028
	40-	108	0.265 ± 0.010*	17	0.482 ± 0.033
	50-	113	0.274 ± 0.009*	7	0.365 ± 0.041
	60-	61	0.282 ± 0.014		
	70-	43	0.265 ± 0.019		

[a] Data from a previous study in our laboratory using a similar study design (ref. 17).

* $P < 0.01$, compared with corresponding controls.

Table 2. Liver Dihydropyrimidine Dehydrogenase (DPD) Activity in Healthy Donors

Group		n	Liver DPD Activity (nmol/min/mg protein)			p Value
			Mean ± S.E.	Highest	Lowest	
Total		138	0.369 ± 0.015	0.894	0.049	
Women		55	0.421 ± 0.024	0.894	0.059	0.005
	African American	10	0.389 ± 0.052			
	Caucasian	45	0.428 ± 0.028			
Men		83	0.335 ± 0.018	0.743	0.049	
	African American	24	0.309 ± 0.033			
	Caucasian	59	0.346 ± 0.021			
African American		34	0.333 ± 0.028	0.689	0.116	0.157
Caucasian		104	0.382 ± 0.017	0.894	0.049	
Age (yr)	20-	12	0.335 ± 0.048	0.667	0.059	0.150
	30-	20	0.426 ± 0.046	0.859	0.049	
	40-	29	0.399 ± 0.026	0.635	0.136	
	50-	24	0.374 ± 0.041	0.894	0.116	
	60-	28	0.299 ± 0.031	0.744	0.071	
	70-	25	0.382 ± 0.032	0.743	0.167	

Table 3. Statistical (ANOVA) Analysis of Liver Dihydropyrimidine Dehydrogenase (DPD) Activity in Healthy Donors

Factors	Group	F Value	p Value	Conclusion
Gender	2	8.33	0.005	Liver DPD activity was significantly higher in women than that in men.
Race	2	2.02	0.157	No significant difference.
Age	6	1.65	0.150	No significant difference.
Gender and Race	2 X 2			Cross analysis by race and gender indicated that liver DPD activity is not race-dependent while gender difference remains.
Gender		7.46	0.007	
Race		2.12	0.148	
Gender and Age	2 X 6			Cross analysis by age and gender indicated that DPD activity is not age-dependent while gender difference remains.
Gender		7.36	0.008	
Age		1.73	0.133	
Race and Age	2 X 6			Cross analysis by age and race DPD activity is neither age- nor race-dependent.
Race		1.96	0.164	
Age		1.67	0.147	
Gender, Race, and Age	2 X 2 X 6			Cross analysis by race and gender indicated that DPD activity is not race-dependent while gender difference remains.
Gender		6.51	0.011	
Race		2.04	0.156	
Age		1.74	0.131	

Table 4. Dihydropyrimidine Dehydrogenase Activity in Normal Liver and Hepatoma Tissues

Group		n	DPD Activity (nmol/min/mg protein)					
			Normal Liver			Hepatoma Tissue		
			Mean ± S.E.	Highest	Lowest	Mean ± S.E.	Highest	Lowest
Total		50	0.45 ± 0.02	0.85	0.19	0.34 ± 0.03	1.45	0.02
Men		43	0.45 ± 0.02	0.85	0.19	0.35 ± 0.03	1.45	0.07
Women		7	0.38 ± 0.02	0.45	0.28	0.31 ± 0.08	0.70	0.02
Age (yr)	30-	8	0.47 ± 0.06	0.73	0.32	0.22 ± 0.03	0.34	0.11
	40-	24	0.44 ± 0.03	0.78	0.19	0.38 ± 0.06	1.45	0.02
	50-	9	0.43 ± 0.06	0.85	0.19	0.34 ± 0.05	0.61	0.15
	60-	9	0.44 ± 0.06	0.80	0.22	0.36 ± 0.06	0.63	0.06

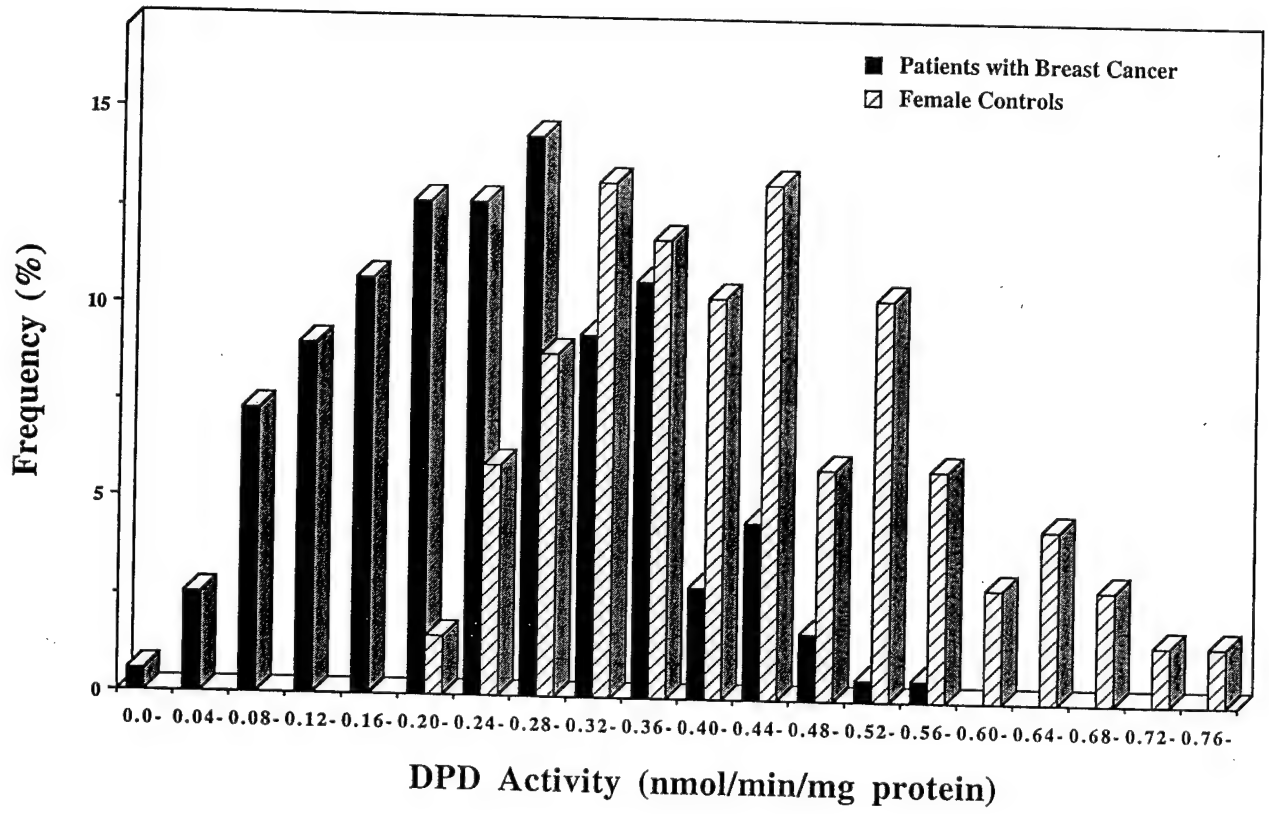

Fig. 1 **Population Distribution of DPD in 360 Patients with Breast Cancer, with Comparison with the General Population**

Statistical analysis demonstrated that PBM DPD activity follows a normal distribution (Guassian distribution).

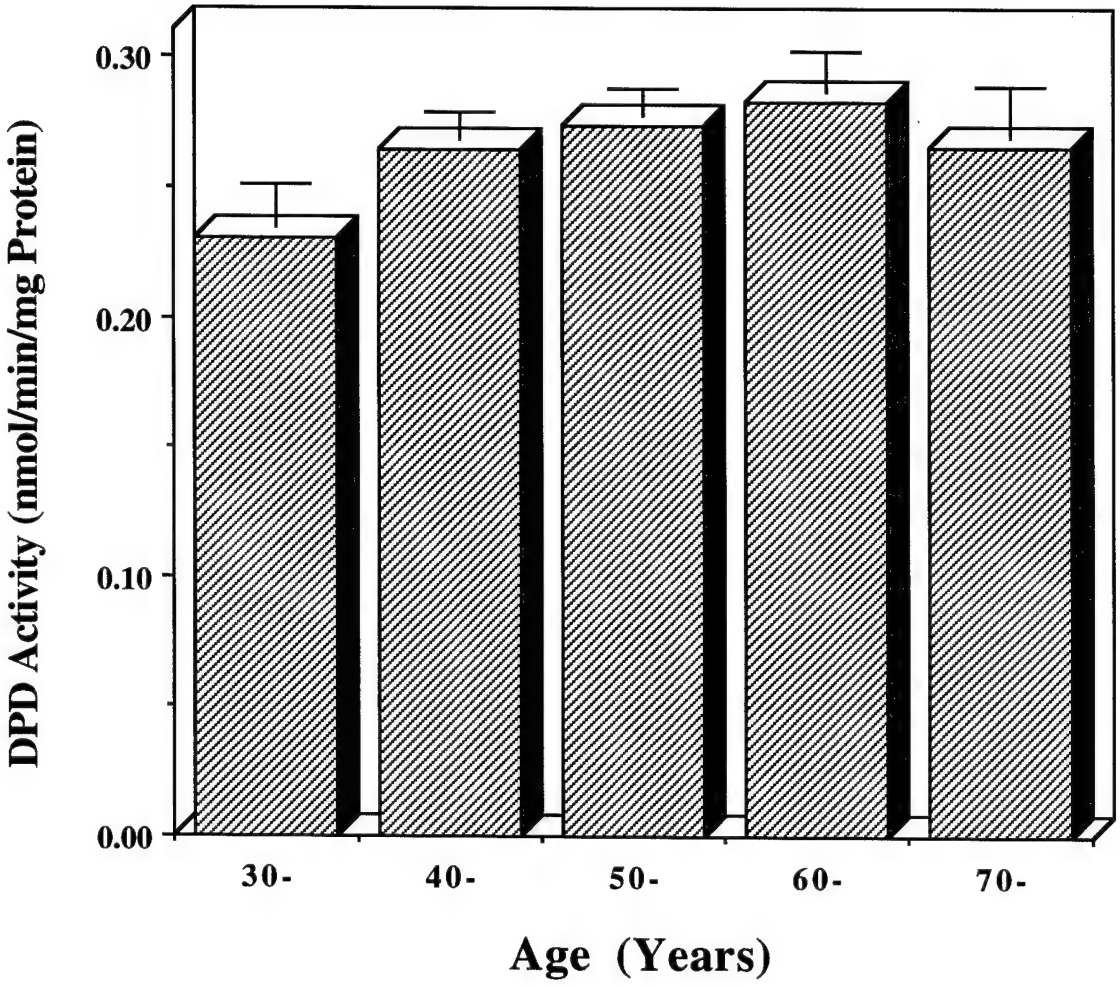

Fig. 2 Comparison of Dihydropyrimidine Dehydrogenase Activities in Patients with Breast Cancer with Different Age.

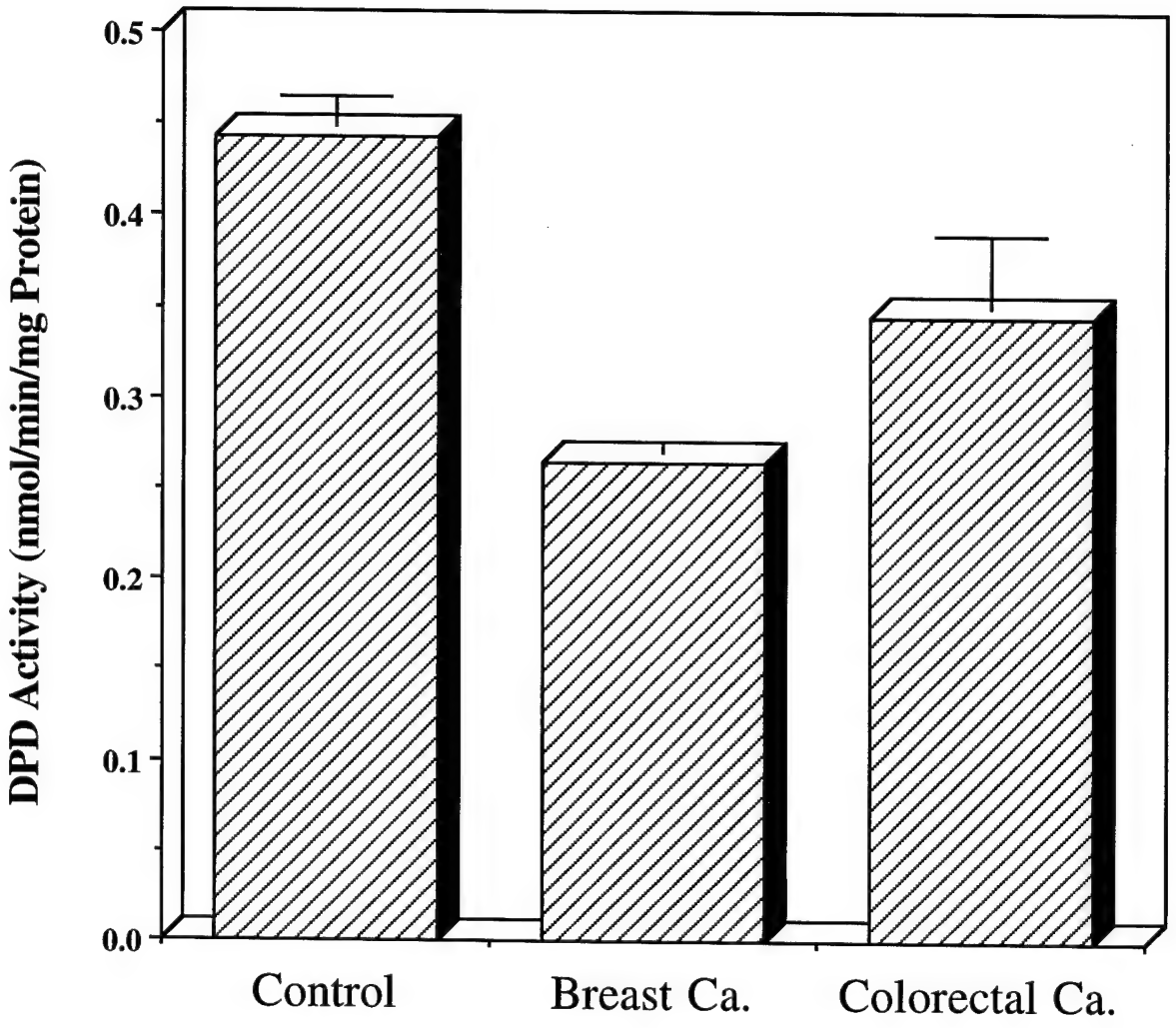

Fig. 3 **Comparison of Dihydropyrimidine Dehydrogenase Activities in Normal Volunteer, Patients with Breast Cancer and Patients with Colon Rectal Cancer**

Statistical analysis demonstrated that there are significant differences:

Breast cancer vs. female control, $p<0.01$;

breast cancer vs. colon rectal cancer, $p<0.05$.

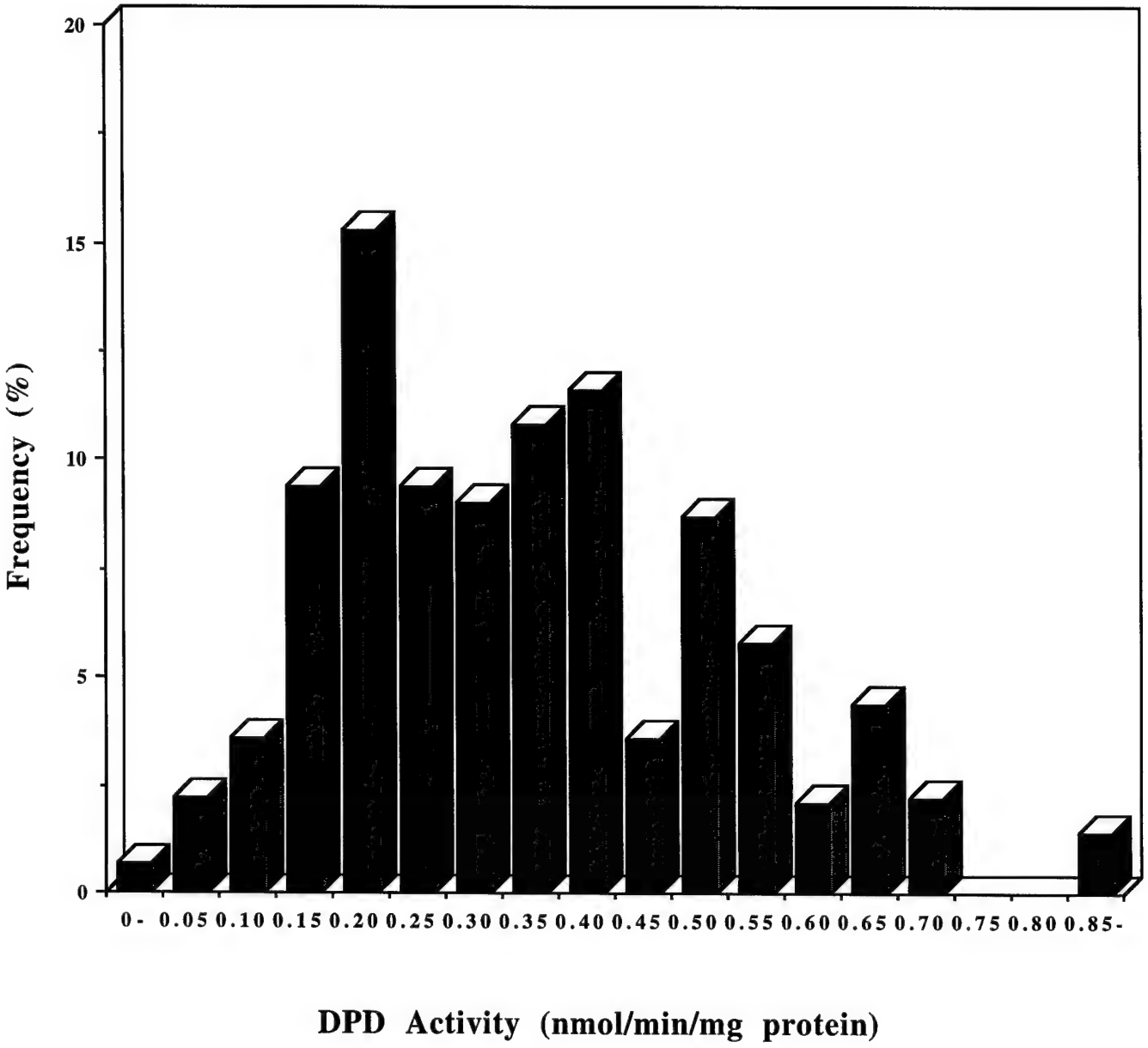

Fig.4 **Population Distribution of Liver Dihydropyrimidine Dehydrogenase Activity**

Statistical analysis demonstrated that liver dihydropyrimidine dehydrogenase activity follows a normal distribution (Guassian distribution).

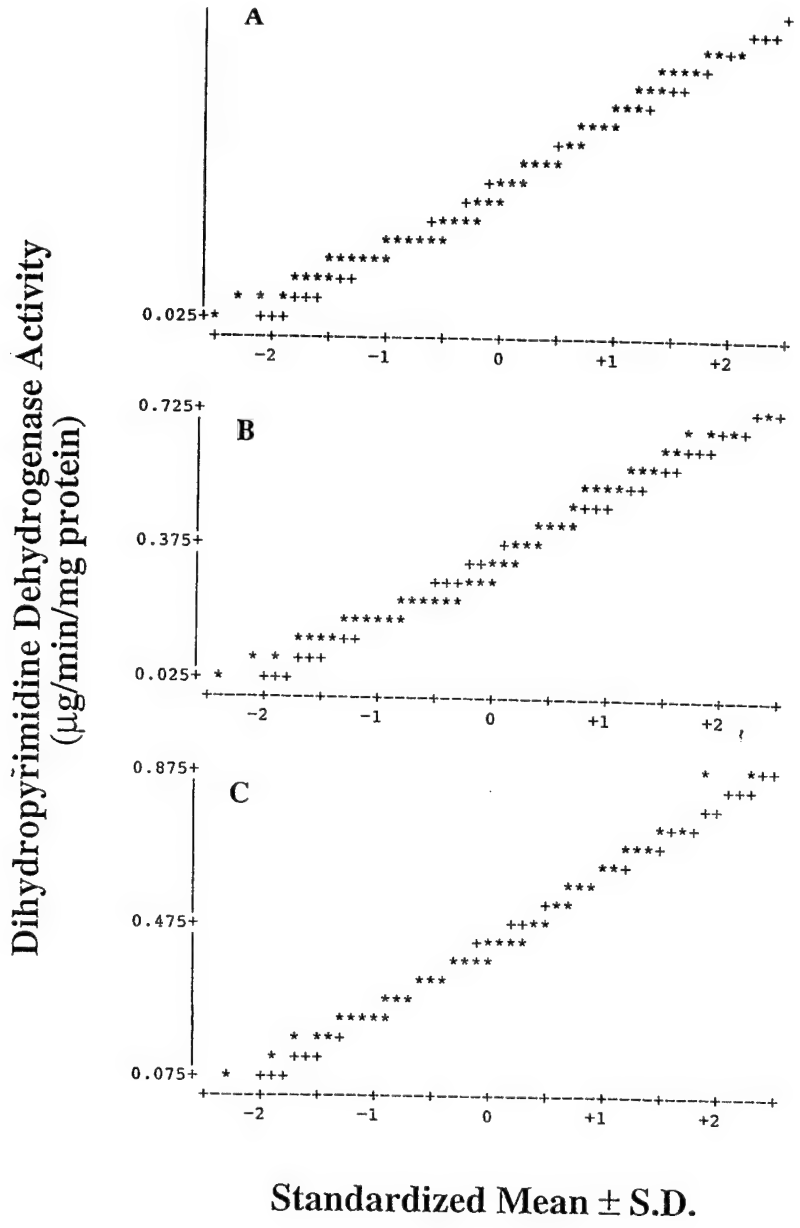

Fig. 5 **Probability Testing of Distribution of Liver Dihydropyrimidine Dehydrogenase Activity**

Panel A: Total subjects; Panel B, male subjects; Panel C, Female subjects.

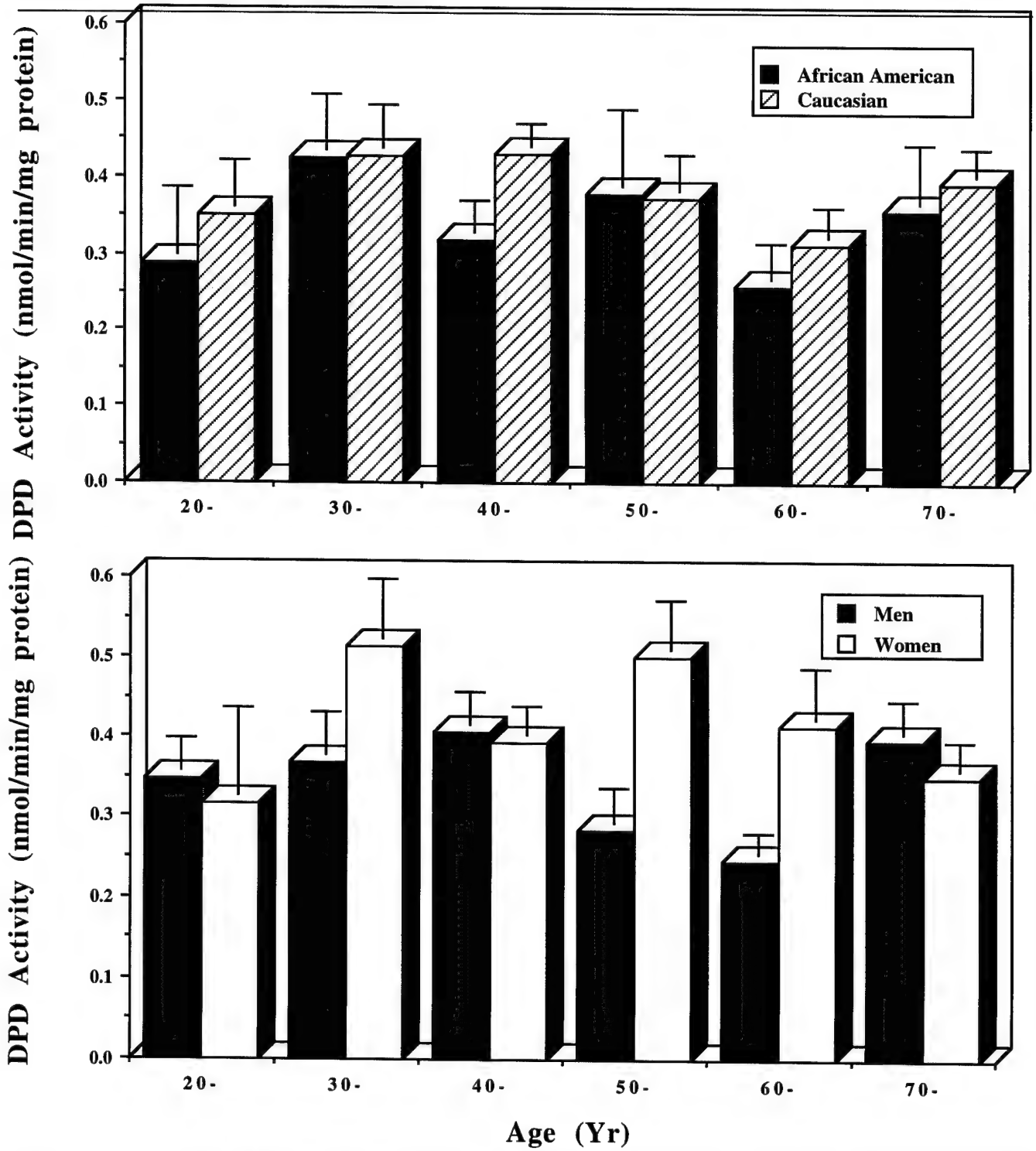

Fig. 6 **Population Distribution of Liver Dihydropyrimidine Dehydrogenase Activity by Age.** Top panel illustrates the comparison of mean (± S.E.) DPD activities between african americans and caucasians, and bottom panel presents the comparison of mean (± S.E.) DPD activities between males and females.

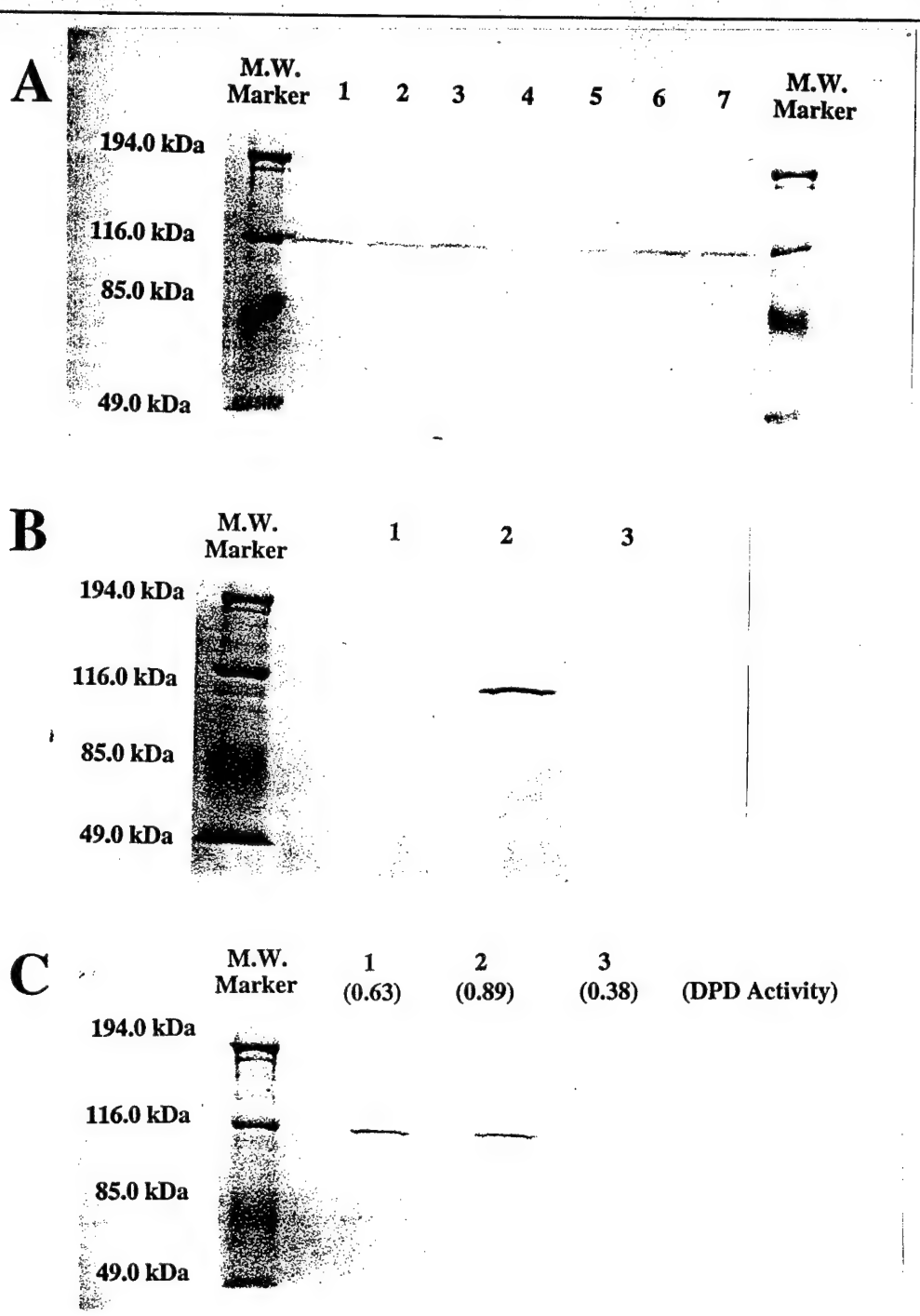

Fig. 7 **Immunoblot Analysis of Liver Dihydropyrimidine Dehydrogenase**
Each lane contains 200 µg of crude liver cytosol. *Panel A*: 7 representative samples with DPD activities between 0.30 and 0.55 µg/min/mg; *Panel B:* Two samples with extremely low DPD activities (< 0.05 µg/min/mg); *Panel C:* Three samples with DPD activities being 0.38, 0.63, and 0.89 µg/min/mg. The correlation between the DPD activity and the DPD protein band density on western blot analysis is illustrated in Figure 8.

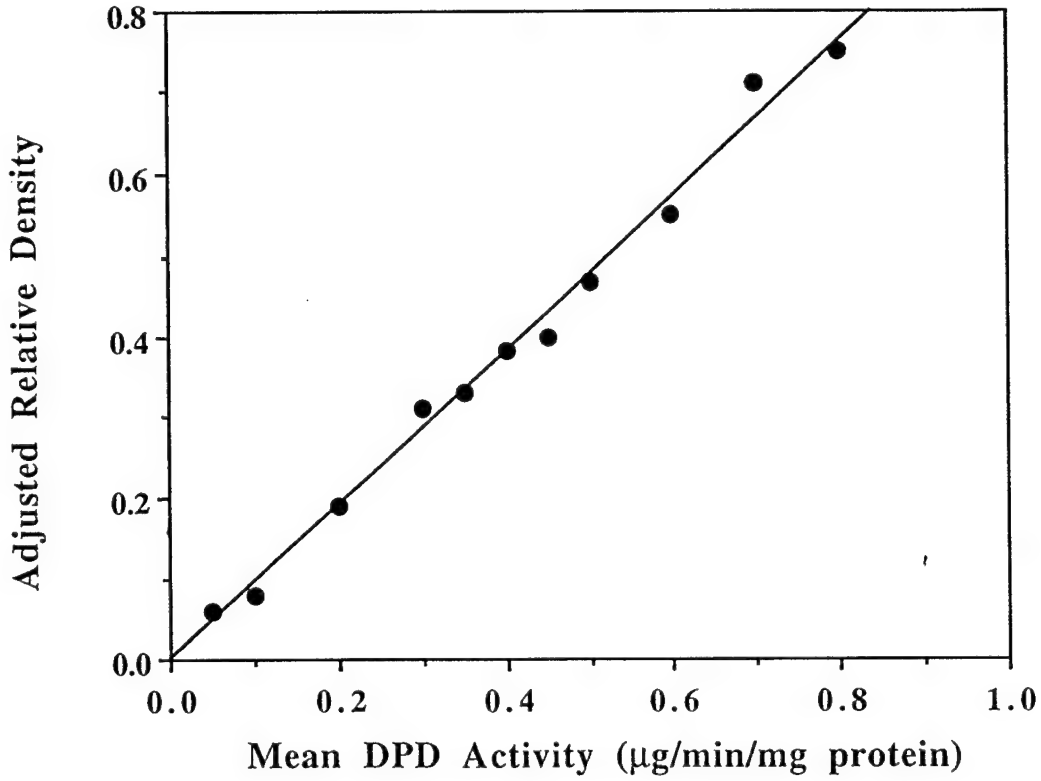

Fig. 8 **Correlation Analysis of DPD Activity and DPD Protein Band Density on Immunoblot Analysis**

Linear correlation analysis indicates that there is a correlation between liver DPD activity and the density of DPD protein on the immunoblot analysis (R^2=0.992, $p<0.01$).

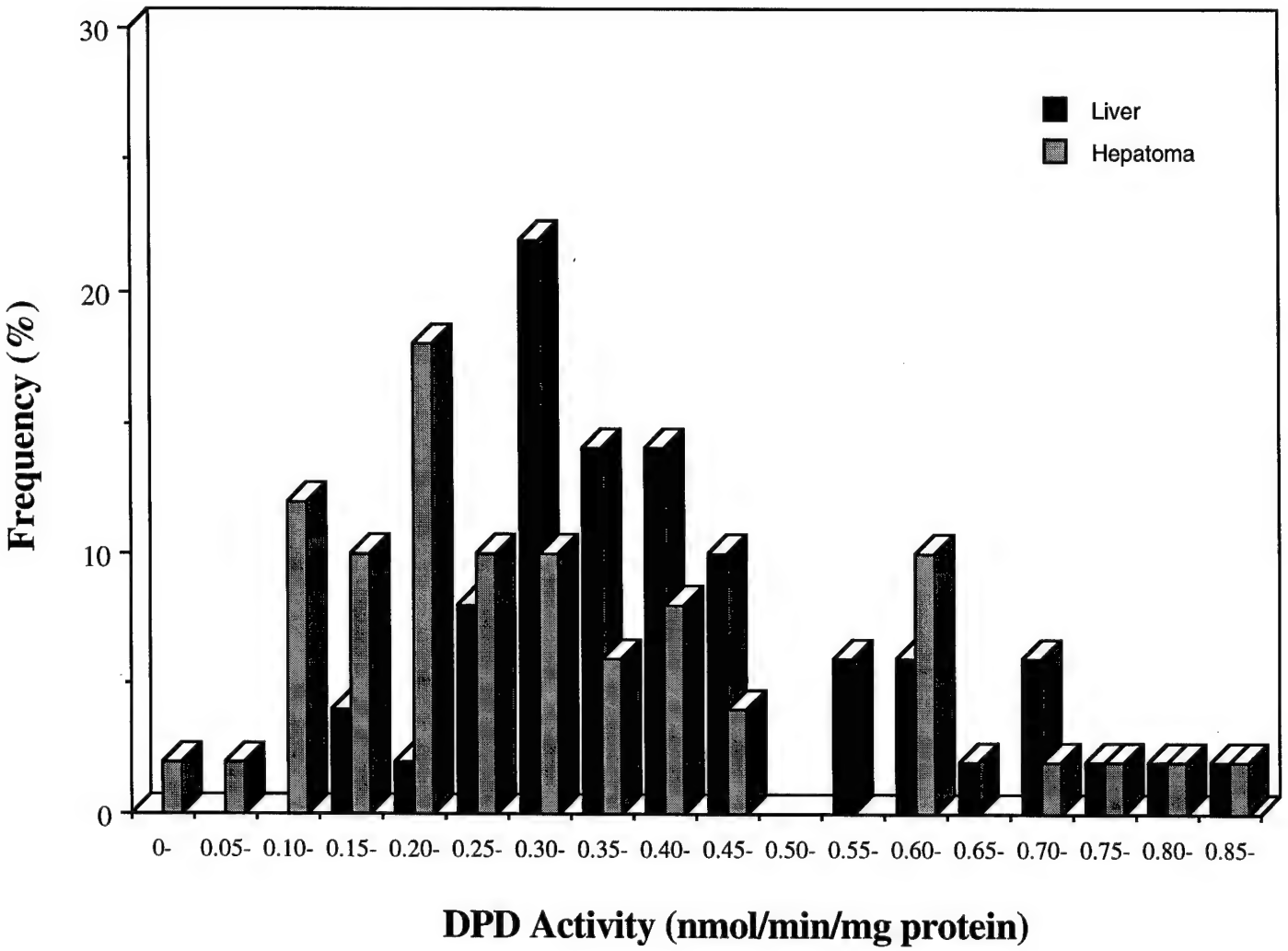

Fig. 9 **Population Distribution of Dihydropyrimidine Dehydrogenase Activity in Normal Liver and Hepatoma**

Statistical analysis demonstrated that dihydropyrimidine dehydrogenase activities follow a normal distribution (Guassian distribution).

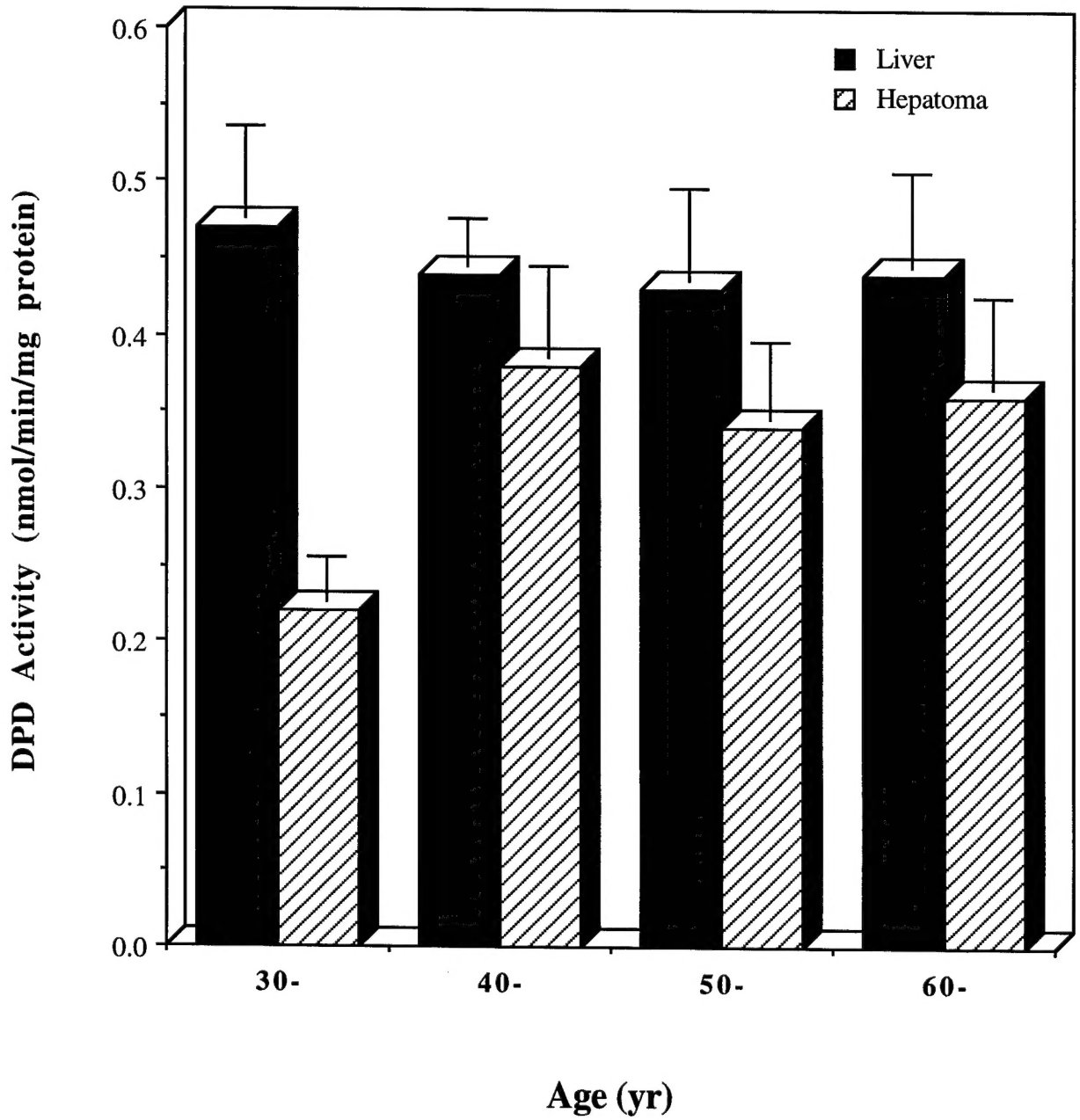

Fig. 10 **Population Distribution of Liver and Hepatoma Dihydropyrimidine Dehydrogenase Activity by Age**

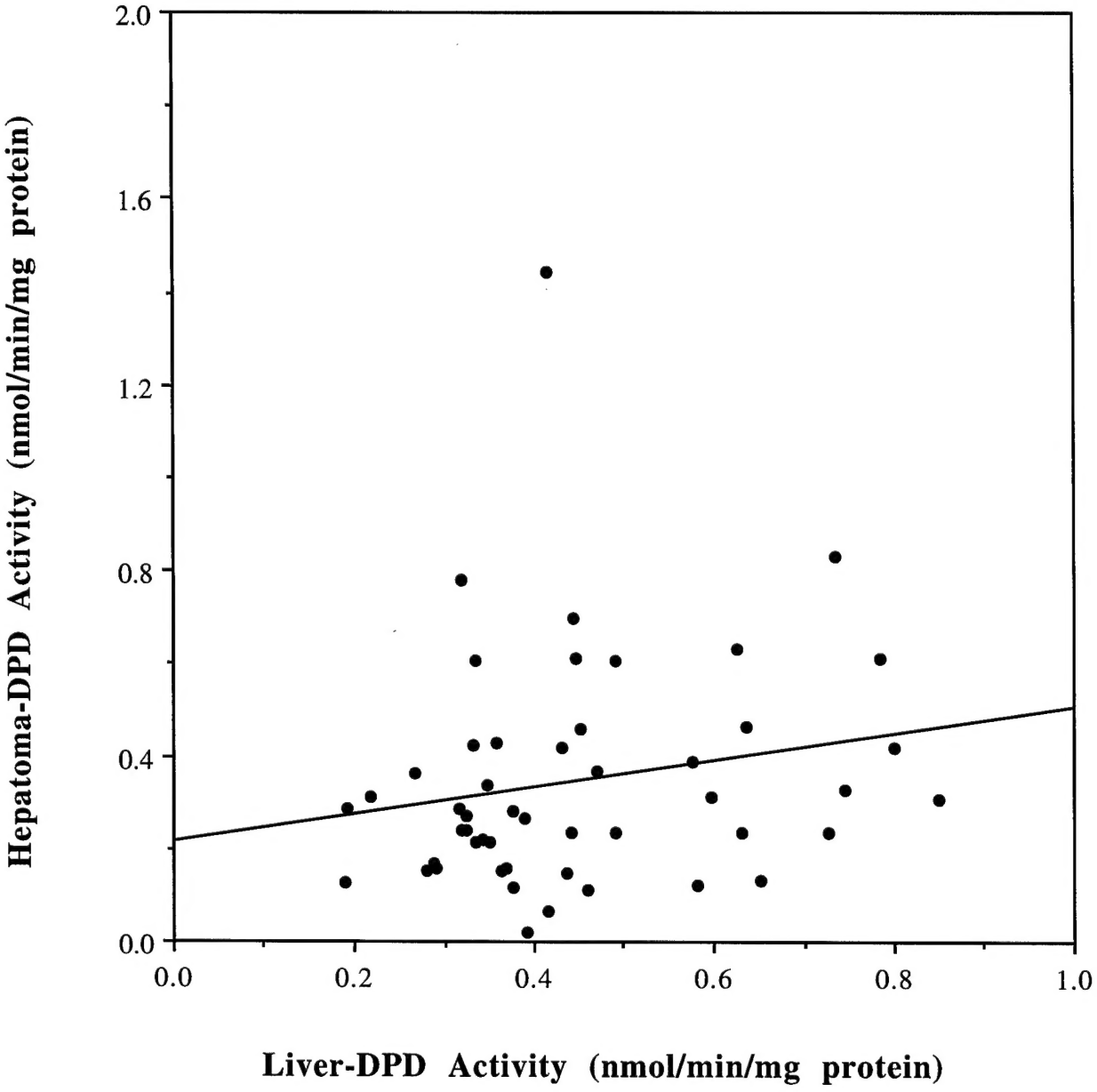

Fig. 11 **Correlation Analysis between Normal Liver DPD Activity and Hepatoma DPD Activity**

APPENDIX

I. LIST OF PUBLICATIONS AND PRESENTATIONS RELATED TO THIS GRANT

Original Papers

1. **Lu, Z.**, Zhang, R., Diasio, R.B. Population characteristics of hepatic dihydropyrimidine dehydrogenase activity, A key metabolic enzyme in 5-fluorouracil chemotherapy. Clin. Pharmacol. Ther. 58: 512-522,1995.

2. Diasio, R.B., Van Kuilenburg, A.B.P., **Lu, Z.**, Zhang, R., Van Lenthe, H., Bakker, H.D., Van Gennip, A.H. Determination of dihydropyrimidine dehydrogenase (DPD) in fibroblasts of a DPD deficient pediatric patient and family members using a polyclonal antibody to human DPD. In: Purine and Pyrimidine Metabolism in Man VIII, Edited by Sahota, S., and Taylor, M., Plenum Press, New York, pp. 7-10, 1995

3. Takimoto, C., **Lu, Z.**, Zhang, R., Liang, M., Larson, L., Grem, J.L., Allegra, C.L., Diasio, R.B., Chu, E. Severe Neurotoxicity following 5-fluorouracil-based chemotherapy in a patient with dihydropyrimidine dehydrogenase deficiency. Clin Cancer Res. 1996; 2:477-481.

4. Jiang, W., **Lu, Z.**, He, Y., Diasio, R.B. Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma; implication for 5-fluorouracil-based chemotherapy. Clin Cancer Res. 1997; 3: 395-399.

5. **Lu, Z.,** Zhang, R., Carpenter, J., Diasio, R.B. Population characteristics of dihydropyrimidine dehydrogenase activity in breast cancer patients; relevance to 5-fluorouracil chemotherapy. Clin Cancer Res.(Submitted).

Book Chapters/Invited Review

1. Diasio, R.B., **Lu, Z.** Dihydropyrimidine Dehydrogenase and 5-Fluorouracil Chemotherapy. J Clin Oncol. 1994; 12: 2410-2414.

2. Diasio, R.B., **Lu, Z.**, Zhang, R, Shihanian, H. Fluoropyrimidine Catabolism. In: Concepts, Mechanisms, and New Targets for Chemotherapy (Muggia F, ed.), Kluwer Academic Publishers, Boston, MA, 1995, pp. 71-93.

3. **Lu, Z.**, Diasio RB. Polymorphic Drug-Metabolizing Enzymes. In: Principles of Antineoplastic Drug Development and Pharmacology (Schilsky, R. L., Milano, G. A., and Ratain, M. J., eds.), Marcel Dekker, New York, NY, 1996, pp. 281-305.

Abstracts/Presentations

1. Amin, G., Shahinian, H., Miller, D., Vukelj, S., Zhang, R., **Lu, Z.**, Diasio, R.B. Severe neurotoxicity following 5-fluorouracil (FUra) chemotherapy in patients with dihydropyrimidine dehydrogenase (DPD) deficiency. Pro. Am. Soc. Clin. Oncol. 1995; 14: 169 (Abstract # 361).

2. Takimoto, C., **Lu, Z.**, Zhang, R., Liang, M., Larson, L., Grem, J.L., Allegra, C.L., Chu, E. Severe neurotoxicity following 5-fluorouracil-based chemotherapy in a patient with dihydropyrimidine dehydrogenase deficiency.Clin. Pharm. Ther. 1996; 59: 161.

3. **Lu, Z.**, Zhang, R., Carpenter, J., Yan, J., Diasio, R.B. Decreased dihydropyrimidine dehydrogenase (DPD) activity in breast cancer patients; Potential increased risk for 5-fluorouracil toxicity. Proc. Am. Assoc. Cancer Res. 1996; 37: 184. (1996 Glaxo Wellcome Oncology Clinical Research Scholar Award- American Association For Cancer Research).

II. LIST OF PERSONNEL RECEIVING PAY FROM THIS EFFORT

Zhihong Lu, M.D., Ph.D., Post-doctoral Fellow/Research Associate, 1994-1997